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**KAHRAMANMARAŞ SÜTÇÜ İMAM UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCE**

**ISOLATION OF *SALMONELLA* FROM CHICKEN
MEATS IN NORTHERN IRAQ**

ABDULREZZAQ HAJI HAJI

**MASTER THESIS
DEPARTMENT OF BIOENGINEERING AND SCIENCES**

KAHRAMANMARAŞ, 2014

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Thesis submitted in candidature for

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KUZEY IRAK'TA ÜRETİLEN TAVUK ETLERİNDE *SALMONELLA* İZOLASYONU

(YÜKSEK LİSANS TEZİ)

Abdulrezzaq Haji HAJI

ÖZET

Bu çalışmada, Duhok ve Erbil'deki lokal market ve kasaplardan temin edilen taze ve dondurulmuş tavuk etlerinde *Salmonella* yaygınlığı araştırılmıştır. Bu amaçla, 50 taze ve 50 dondurulmuş tavuk eti kültürel, biyokimyasal ve serolojik yöntemlerle incelenmiştir. Ayrıca, elde edilen *Salmonella* izolatlarının 13 farklı antibiyotiğe hassasiyetleri ölçülmüştür. Uygulan kültürel metotta, 100 örnekten 11'inde muhtemel *Salmonella* kolonileri görülmüştür. Bunlardan 9 tanesinin taze (%18), 2 tanesinin de (%4) dondurulmuş tavuk etlerinden elde edilmiştir illere göre, 7 tanesinin (%15,6) Erbil'den, 4 tanesinin de (%7.3) Duhok'tan toplanan tavuk etlerindise ileri olduğu bulunmuştur. *Salmonella* şüphesi bulunan 11 kültür daha sonra biyokimyasal doğrulama testlere tabi tutulmuş ve geleneksel metotlarla yapılan testlerde 8'inin ve RapID™ ONE sistemi ile yapılan testte ise 11'in de *Salmonella* olduğu görülmüştür. Somatik (O) ve flagellar (H) antiseralar ile yapılan serolojik testlerde, *Salmonella* şüphesi bulunan 11 kültür aglütinasyon-pozitif bulunmuştur. Irak Merkez Laboratuvarında yaptırılan serotip çalışmasında, 8 kültürde *S. typhimurium*, 3 kültürde ise *S. anatum* olduğu bulunmuştur. Her iki serotipteki bakteriyel izolatların 13 antibiyotikten sadece 4 tanesine (CT, C, CIP ve TE), ayrıca *S. anatum*'un K ve TMP 'ye de duyarlı olduğu görülmüştür. Sonuç olarak, Erbil'de üretilen tavuk etlerinde *Salmonella* kontamasyonu daha fazla görülmüş olup, *S. typhimurium* *S. anatum*'a göre daha dominant olduğu ve her iki serotipinde birden çok antibiyotiğe karşı direnç gösterdiği bulunmuştur.

Anahtar Kelimeler: Tavuk eti, *Salmonella*, geleneksel metotlar, RapID™ ONE test, serolojik test, antibiyotik direçliliği.

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ISOLATION OF *SALMONELLA* FROM CHICKEN MEATS IN NORTHERN IRAQ

(M.Sc. THESIS)

Abdulrezzaq Haji HAJI

ABSTRACT

The present study was conducted to isolate and confirm *Salmonella* in fresh and frozen chicken meats collected in the local markets and butcheries in Duhok and Arbil, Northern Iraq. In this respect, fifty fresh and fifty frozen chicken meats were analyzed by the cultural, biochemical and serological methods. The suspected cultures were later subjected to the antibiotic resistance test using 13 known antibiotics. The cultural method for the isolation of *Salmonella* indicated that 11 agar plates 9 from fresh (18%) and 2 from frozen (4%) were probably suspected with *Salmonella*. Seven suspected meat samples was collected in Arbil (15.6%) and 4 in Duhok (7.3%). The biochemical confirmation tests performed later confirmed that 8 of 11 suspected culture contained *Salmonella*. The RapID™ ONE test for further confirmation showed that all 11 suspected cultures probably infected with *Salmonella* with two different species. Using somatic (O) and flagellar (H) antisera, it was observed agglutination of the suspected cultures with polyvalent O and H antisera, indicating the suspected cultures contained *Salmonella*. The cultures were serotyped by the Central Health Laboratory, Iraq, and confirmed that 8 of them were infected with *S. typhimurium*, and 3 of them with *S. anatum*. *S. typhimurium* and *S. anatum* were found to be sensitive only to CT, C, CIP and TE. *S. anatum* also exhibited sensitivity to K and TMP. These results indicated that the prevalence of *Salmonella* is higher in Arbil than in Duhok; *S. typhimurium* is dominant compared to *S. anatum* in the meat samples and both show multi-drug resistance.

Keywords: Chicken meat, *Salmonella*, conventional methods, RapID™ ONE test, serological test, antibiotic resistance.

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1. INTRODUCTION

Illnesses from food are one of the most important economic and health problems among industrial and non-industrial countries. In recent years, *Salmonella* has been one of the most common causes of food born disease (Busani *et al.*, 2006). Salmonellosis is an infectious disease which often occurs through contaminated food, especially food products with an animal origin such as meat, chicken, egg, animal foods and sometimes vegetables in the food chain (Bouchrif *et al.*, 2009). *Salmonella* lives in the intestinal track of humans and other animals, including birds. Poultry is the largest source of *Salmonella* for humans (Lynch *et al.*, 2006).

Poultry meat is contaminated with *Salmonella* not only by infected poultry, but also by cross-contamination with feces, water, instruments and worker's hands during the slaughter process and handling. Chicken might thus provide the main transmission route of infection, especially with the increasing consumer demand for this food. Poultry and poultry products are frequently contaminated with *Salmonella* that can be transmitted to humans through the handling of raw poultry carcasses and products, or through consumption of undercooked poultry meat (Bailey and Cosby, 2003; Kimura *et al.*, 2004).

Salmonella species are recognized as very important food-borne, water-borne organisms and the cause of a significant range of illnesses including food poisoning (gastroenteritis), typhoid (enteric fever), paratyphoid, bacteraemia, septicemia with different variety of sequelae (Bell and Kyriakides, 2002). *Salmonella* spp. are estimated to cause more than 30% of all bacterial foodborne deaths (Mead *et al.*, 1999).

There is an increasing trend in production and consumption of meat, poultry meat and their products. This, of course, requires adequate control and inspection both during poultry rearing and in slaughterhouses, processing plants and shops. Consumers are also a link in the chain of food-borne human diseases, because of the way they store and cook meat, poultry meat, and their products (Kozacinski *et al.*, 2006). Therefore, microbial safety of food is a significant concern of consumers and industries today. Because of the extensive use of antimicrobial agents in animal and human therapy, resistance became a public health concern (Sefton, 2002). Since the beginning of 1990, salmonella strains which are resistant to arrange of antimicrobials have emerged and are now a serious public health concern (WHO, 2013).

The purpose of the study was to establish the prevalence of *Salmonella* in chicken meats obtained from Northern Iraq. The aims are to detect *Salmonella* in chicken meats in Northern Iraq and check the resistance of *Salmonella* to common antibiotics.

2. LITERATURE REVIEW

Salmonella is a gram-negative, rod-shaped bacillus with flagella that can cause diarrheal illness in humans. They are microscopic living creatures that pass from the feces of humans or animals to other humans or other animals. It has diameter of 0.7-1.5µm and length of 2-5µm. *Salmonella* is the most prevalent foodborne bacteria (Bean *et al.*, 1990) along with many others, having over 2,579 serotypes (Grimont and Weill, 2007). Two types, *S. enteritidis* and *S. typhimurium* are most prevalent serotypes in the world, causing gastroenteritis, and accounting for half of all human infections.

Salmonella is part of normal gut flora but only pathogenic serotypes, when outnumber benign bacteria, cause an infection. Strains that show no symptoms in animals can make humans sick, and vice versa. If present in food, it does not usually affect the taste, smell, or appearance of the food. The bacteria live in the intestinal tracts of infected animals and humans. *Salmonella*, discovered by Dr. Daniel E. Salmon in 1885, have been known to cause illness for over 100 years.

There are three main ways *Salmonella* can enter the food supply to cause illness:

1) Animals harbor *Salmonella*, making meats, poultry, eggs, and milk often implicated vehicles.

2) *Salmonella*, which are introduced into the environment, possibly through manure and litter, may persist and contaminate fruits and vegetables on the farm.

3) Cross-contamination in the food service environment or the home, often between raw poultry and ready-to-eat (RTE) products, such as raw vegetables, can also cause salmonellosis.

Salmonella lives in the intestinal track of humans and other animals, including birds. Poultry is the largest source of *Salmonella* for humans (Lynch *et al.*, 2006). *Salmonella* is usually transmitted to humans by eating foods contaminated with animal feces. *Salmonella* present on raw meat and poultry could survive if the product is not cooked to a safe minimum internal temperature, as measured with a food thermometer. It grows at temperatures between 5°C and 45°C and can be readily destroyed by cooking to 73°C. *Salmonella* cannot grow at refrigerator or freezer temperatures. They do survive

refrigeration and freezing, however, begin to grow again once warmed to room temperature. *Salmonella* can also cause food-borne illness through cross-contamination, e.g., when juices from raw meat or poultry come in contact with ready-to-eat foods. Unwashed hands of an infected food handler may also contaminate food. *Salmonella* can also be found in the feces of some pets, especially those with diarrhea. People can become infected if they do not wash their hands after contact with these feces (Jay *et al.*, 2005).

Some *Salmonella* are host-adapted like *S. pullorum* in poultry, *S. dublin* in cattle; however, most are not. Although any *Salmonella* is a potential pathogen for humans, most foodborne salmonellosis is caused by non-host-adapted serotypes. The strain, *S. enterica* subsp. *enterica* serotype typhimurium DT 104, are resistant to several antibiotics, and the increase in its prevalence poses challenges in treatment of the infection (Glynn *et al.*, 1998).

Some infected individuals may excrete *Salmonella* for weeks, months, and, occasionally, years with little or no evidence of clinical disease. Improper hygiene practices by these individuals may lead to either contamination of foods or direct person-to-person contamination. Contamination of the products can occur pre-processing (due to lack of hygiene and proper processing conditions) and post-processing (due to mishandling and lack of proper storage) (Myint, 2004)

2.1. Classification of *Salmonella*

Salmonella belongs to the family Enterobacteriaceae (Jay *et al.*, 2005). The genus *Salmonella* contains two species; *S. enterica* and *S. bongori* (Table 2.1), which was formerly subspecies V. Six subspecies are differentiated within *S. enterica* based on their biochemical and genomic characteristics, a Roman numeral and a name are used for the designation of these six subspecies as follows: I. *S. enterica* subsp. *enterica*; II. *S. enterica* subsp. *salamae*; III. *S. enterica* subsp. *arizonae*; IV. *S. enterica* subsp. *diarizonae*; V. *S. enterica* subsp. *houtenae*, and VI. *S. enterica* subsp. *indica* (Brenner *et al.*, 2000). With regard to food safety *S. enterica* subsp. *enterica* is the subspecies of most concern because the strains within these serogroups are known to cause 99% of *Salmonella* infections in humans (Bell and Kyriakides., 2002).

Table 2.1. Species and subspecies in the *salmonella* genus (Grimont and Weill, 2007)

Species	Subspecies	Number of serovars
<i>S. Enteric</i>	Enterica	1531
	Salamae	505
	Arizonae	99
	Diarizonae	336
	Houteane	73
	Indica	13
<i>S. Bongori</i>		22
Total		2579

By newer convention, names are retained only for subspecies *enterica* serovars, and these names are no longer italicized. The first letter is a capital letter “S” followed by the serovar names of subspecies *enterica* (e.g. *typhimurium* or *montevideo*). At the first citation of the serotype the genus name is given followed by the word “serotype” or the abbreviation “ser.” followed by the serotype name. This project follows the abbreviated modern naming system, i.e. *S. typhimurium* rather than the more complete nomenclature *S. enterica*, subsp. *enterica* serovar *typhimurium*. (Molbak *et al.*, 2006).

Radostits *et al.* (2000) and Jay *et al.* (2005) reported that the genus *Salmonella* can be roughly classified in three groups according to their adaptation to hosts:

Group 1: Those that infect human only: they include *S. typhi*, *S. paratyphi* A, *S. paratyphi* C, this group includes the agents of typhoid and paratyphoid fevers.

Group 2: The host-adapted serovars (some of which are human pathogens and may be contracted from foods): *S. gallinarum* (poultry), *S. dublin* (cattle), *S. abortusequi* (horse), *S. abortusovis* (sheep) and *S. choleraesuis* (swine).

Group 3: Unadapted serovars (no host preference): they are pathogenic for humans and other animals, and include most foodborne serovars (*S. typhimurium*, *S. enteritidis*, etc)

The molecular mechanisms that are responsible for a host restriction of some serotypes are poorly understood, but most believe there is a repertoire of adhesin molecules expressed by *Salmonella* that determines the host surface recognition (Baumler *et al.*, 1998). *Salmonella* may have evolved host specificity through a series of gene transfers and deletions. Studies in pigeons of the host adapted serovars *S. typhimurium* DT2 and DT99 demonstrate the point mutation or small deletions in the genome (Andrews-Polymenis *et al.*, 2004), others have demonstrated that the host-adapted serotypes like *S. typhi* possess genomic insertions that may promote host restriction (Liu *et al.*, 2006).

2.2. Serotype of *Salmonella*

Serotyping has become an important tool for the national *Salmonella* surveillance system to tract *Salmonella* isolates for epidemiological surveillance and to investigate disease outbreaks (CDC, 2005).

According to the White-Kauffmann-Le Minor scheme, subspecies are further divided into serovars (serotypes) by serologic testing with monovalent antisera. Serotyping is widely used as an epidemiological and standardized typing method for *Salmonella*. Approximately 99% of the 2579 currently known serovars composes the genus belong to *S. enterica* (Grimont and Weill, 2007). The DNA sequence similarity between serovars is 96-99% (Edwards *et al.*, 2002). Serotyping is based on the antigenic variability at lipopolysaccharide (O-antigen), the phase-1 and phase-2 flagellin (H1- and H2-antigen), as well as for some serovars on the capsular polysaccharide (Vi-antigen) (Table 2.2). Altogether 67 different serogroups defined by O-antigens, classified by the White-Kauffmann-Le Minor scheme are known (Grimont and Weill, 2007).

A serogroup comprises serotypes with similar O antigens. Lysogenisation by certain converting bacteriophages may produce the change in the O antigen, for example in serogroup E, a phage can alter the O antigen 3,10 to 3,15 thus changing *S. anatum* to *S. newington* (Quinn *et al.*, 2002).

Serovars are designated by their antigenic formulae (O-antigen:H1-antigen:H2-antigen) following the subspecies name. Serovars belonging to *S. enterica* subspecies I are designated with a name which is related to the geographical place where the serovar was first isolated (Grimont and Weill, 2007).

Table 2.2. Antigens of some *Salmonella* serotypes (Jay, 1992; Jay *et al.*, 2005).

Serotypes	Serogroups	Somatic antigens(O)	Flagella(H) antigen	
			Phase 1	Phase 2
<i>S. paratyphi A</i>	A	1, 2,12	A	(1,5)
<i>S. typhimurium</i>	B	1,4,(5),12	I	1,2
<i>S. agona</i>	B	4,12	f, g, s	-
<i>S. derby</i>	B	1,4, (5),12	f, g	(1, 2)
<i>S. abortusovis</i>	B	4,12	C	1,6
<i>S. Newport</i>	C	8	e, h	1,2
<i>S. typhi</i>	D	9,12, (Vi)	E	1,2
<i>S. enteritidis</i>	D	1,9,12	g, m	(1,7)
<i>S. gallinarum</i>	D	1,9,12	-	-
<i>S. anatum</i>	D	3,10	e, h	1,6
<i>S. newington</i>	E	3,15	e, h	1,6

2.2.1. Somatic (o) or cell wall antigens

The somatic (O) antigens represent the side-chain of repeating sugar units projecting outwards from the lipopolysaccharide (LPS) layer on the surface of the bacterial cell wall. The O antigens are heat stable, being unaffected by heating for 2.5 hours at 100°C and the alcohol resistant, with a standing treatment with 96% ethanol at 37°C for 4 hours and unaffected by 0.2% formaldehyde (Old, 1996). O antigen is a polymer of O sub-units and each O sub-unit consists of four to six sugars. Variation of sugar components of O sub-unit leads to a variation in O antigen (CDC, 2005). The O is designated with Arabic numerals 1, 2, 3, 4, while group classification is based on several of these antigens (Carter *et al.*, 1995; Carter and Wise, 2004).

2.2.2. Flagellar (H) antigens

These antigens represent a determinant group on the flagellar protein. They are heat

labile and alcohol labile, but are well preserved in 0.04-0.2% formaldehyde and detachment of all flagella is achieved by heating for 30 minutes at 100°C. Few *Salmonella* do not have H antigens when they express non motile phase. Most H antigens are diphasic strains which have two different types of H antigens, but some H antigens are monophasic (express one phase of H antigen only) (Old, 1996). Most *Salmonella* have H antigens which can switch between two types known as phase 1 and phase 2. The H antigens of phase 1 are designated by small letters of alphabet a, b, c, and less specific for *Salmonella*. Isolates in this phase before can be typed and those of phase 2 are designated by Arabic numerals 1, 2, 3, 4, which are less specific and duplicated in other bacteria (Carter *et al.*, 1995; Carter and Wise, 2004).

2.2.3. Surface (envelope or capsular; Vi) antigens

Envelope antigens in *Salmonella* may mask O antigens, and the bacteria will not be agglutinated with O antisera. One specific surface antigen is well known; the Vi antigen. The Vi antigen occurs in some *Salmonella* serovars: *typhi*, *paratyphi C*, and rarely in *S. dublin* (Morris *et al.*, 2003). The Vi antigen is heat labile, which can be removed from the bacteria by heating for 1 hour at 100°C (Old, 1996). These antigens may interfere with agglutinability of O antisera (Carter *et al.*, 1995; Carter and Wise, 2004).

Serovars can be phenotypically further sub-typed by a specific phage typing. Human and mammalian animal infections are often caused by few serovars belonging almost exclusively to *S. enterica* subsp. *enterica*. However, other animal classes e.g. reptiles show predominant infections from other subspecies, in this case IIIa (Libby *et al.*, 2002). These very common *S. enterica* subsp. *enterica* serovars cannot be adequately characterised by serotyping alone. Phage typing enables a detailed sub-typing of a certain *Salmonella* serovar. This typing technique infects a strain of a certain serovar with a number of bacteriophages. By this technique a set of defined phages is used to obtain a lysis pattern of the strain. This pattern is associated to a definite phage type. Phage typing is based on the sensitivity of cultures to a series of bacteriophages at appropriate dilution (Timoney *et al.*, 1988). Phage typing has been shown to be very useful in the description of pandemic clones of *Salmonella*, such as *Salmonella* serovar *typhimurium* definitive type104 (DT104) (Prager *et al.*, 1999).

2.3. Food Poisoning

2.3.1. Bacterial food poisoning

The term food poisoning embraces of acute illnesses caused by the ingestion of foods that contain substances or agents injurious to human. These substances may be chemical or biological in origin (Quinn *et al.*, 2002). The occurrence of food poisoning depends on a specific set of circumstances, of which some or all of the following factors may be present:

1. The food is contaminated with bacteria capable of causing food poisoning from human, animal or environmental sources. Cross-contamination from raw to cooked food can occur.
2. The food must be suitable for bacterial multiplication so that the levels of bacteria or bacterial toxins in the food are sufficient to cause food poisoning.
3. The bacterium itself must produce toxins or has other virulence factors that cause clinical signs of food poisoning. It must also have the ability to survive in the food during manufacture, storage, distribution and preparation. This survival is aided by poor techniques such as under cooking.
4. Susceptible humans must eat food containing critical levels of bacteria and/ or bacterial toxins.

Bacterial food poisoning is manifested as an acute gastroenteritis due to the ingestion of bacteria themselves and/or toxins present in food. There are two primary types of foodborne diseases:

1. Intoxications resulting from the consumption of pre-formed toxins in foods, the bacteria responsible for which include *Staphylococcus aureus*, *Bacillus cereus*, *Clostridium botulinum*, *Clostridium perfringens*, Enterotoxogenic *Escherichia coli* producing heat stable toxin and some *Streptococci* are capable of elaborating an enterotoxin in foods.
2. Foodborne infection where bacteria have multiplied in the food and are ingested in relatively large number. They may also multiply in vivo producing enterotoxin in the intestinal tract, bacteria causing these acute foodborne infections include *Salmonella*, *Vibrio parahaemolyticus*, *Campylobacter jejuni*,

Yersinia enterocolitica, *Aeromonas hydrophila*, *Listeria monocytogens* and *Escherichia coli* (Quinn *et al.*, 2002; Prescott *et al.*, 2005).

2.3.2. *Salmonella* food poisoning

This syndrome is caused by the ingestion of food contaminated with significant numbers of non-host specific species or serotypes of the genus *Salmonella* (Jay *et al.*, 2005). The most incriminated foods in outbreaks of human salmonellosis are those of animal origin (D' Aoust, 1991), which usually resulted from infected animals used in food production or from the contamination of the carcasses or the edible organs (Alemayehu *et al.*, 2003). Outbreaks of *Salmonella* food poisoning are most commonly associated with meat, meat products, poultry meat or raw-egg dishes and less commonly with milk, cream, sea foods, salad vegetables and canned meat (Quinn *et al.*, 2002). Food with a high fat content or a good buffering capacity may protect a relatively small dose of *Salmonella* during their passage through the acidic region of the stomach, and thus permitting a lower dose than the normal to initiate food poisoning. Examples of such foods, which have been associated with *Salmonella* food poisoning, are chocolate and cheddar cheese (Quinn *et al.*, 2002).

The clinical picture of *Salmonella* food poisoning is that of acute gastroenteritis consisting of fever, nausea, vomiting, abdominal cramps, headache, diarrhea and dehydration which are typical symptoms and severity ranges from mild pain and little diarrhea to the extreme pain and the bloody severe diarrhea (CDC, 2001). Occasionally, this kind of disease may be developed into more serious septicemic conditions (Humphrey *et al.*, 1998). Typically, symptoms of gastroenteritis develop within 6 to 72 hours after ingestion of the bacteria (Pegues *et al.*, 2005). The symptoms are usually self-limiting and typically resolved within 2 to 7 days (Foley and Lynne, 2008). In humans, the presence and severity of symptoms depends on the infective dose (Humphrey *et al.*, 1998). The infecting dose of *Salmonella* for humans varies with *Salmonella* spp., the type of food substrate and immunological status of human. The dose required to produce signs of food poisoning varies from about 10^3 - 10^8 *Salmonella* (Quinn *et al.*, 2002). Disease can actually be caused by 50-100 cells when consumed as part of contaminated food (Jay *et al.*, 2005). Garbutt (1997) have noted that salmonellosis may be caused by as few as 10 cells.

2.3.3. Incidence of *Salmonella* food poisoning

Salmonella has been considered to be the most important causal agents of foodborne illnesses throughout the world and hundreds of outbreaks of the foodborne salmonellosis still occur in most countries every year (Bell and Kyriakides, 2002). The prevalence of bacterial food poisoning varies from one country to others and depends on such factors as farming practices, type of foods commonly consumed, food hygiene standards and method of food preparation. According to the Centers for Disease Control and Prevention (CDC), roughly 1 to 4 million cases of foodborne salmonellosis occur annually in the United States and an estimated 1.1 million cases of salmonellosis occurred in 2010 in United States, leading to approximately 19,336 hospitalizations and 378 deaths (Scharff, 2012).

The majority of human infection with *Salmonella* occurs through ingestion of contaminated foods and poultry in particular is a more frequent source of *Salmonella* (Mead *et al.*, 1999). *S. enterica* serotypes *enteritidis* are considered the most important group of food borne *Salmonella* causing gastrointestinal illnesses of varying severity in humans (Forshell and Wierup, 2006). In Western Europe, America and Australia, *S. typhimurium* tend to be the most common *Salmonella* spp., involved in food poisoning but in Britain, since in 1998, the reported cases of *S. enteritidis* have exceeded those of *S. typhimurium*. All of the increase has due to *S. enteritidis* phage type PT4 (Quinn *et al.*, 2002).

Between 1987 and 1997, 61% of human cases reported to the Centre of Disease Control and Prevention (CDC) were caused by five serotypes, including *S. enterica* serotype *typhimurium* (23%), *S. enterica* serotype *enteritidis* (21%), *S. enterica* serotype *heidelberg* (8%), *S. enterica* serotype *newport* (5%), and *S. enterica* serotype *hadar* (4%) (Olsen *et al.*, 2001).

The U. S. Department of Agriculture (USDA) estimated that bacteria account for 3.6-7.1 million foodborne illnesses each year. Among the leading causes are *Salmonella*, *Campylobacter* and *Escherichia coli* O157:H7 which colonize the intestines of farm animals and subsequently contaminate meat products during processing (Tortora *et al.*, 2004). According to a survey performed by CDC between 1983 and 1987, poultry and its products was the most common source of human infections which was about 18.2% followed by beef 11.2% and 2.9 from pork (Tauxe, 1991).

2.3.4. Foodborne pathogens associated with poultry products

Although various foods can serve as sources of foodborne illness, poultry and their products are important sources of human disease with a variety of foodborne pathogens. The origin of microbial contamination in poultry is varied: vertical or horizontal transmission on farms and during transport; slaughter and processing, particularly in scalding, defeathering and eviscerating, and separation into giblets, as well as cross contamination during deboning, slicing, chopping, grinding, and preparation for direct consumption at home (Kusumaningrum *et al.*, 2004).

In general, the presence of small numbers of pathogens in raw materials is not a problem because meat and poultry are normally cooked before consumption (James and James, 2002). It is generally agreed that the internal tissues of healthy slaughter animals are free of bacteria at the time of slaughter. However, under the current practices of meat and poultry processing, it is impossible to guarantee sterility of the final products (Jay *et al.*, 2005). The main bacterial hazards of concern in meat and poultry include infectious Gram-negative bacteria such as *Salmonella*, *Campylobacter* spp., Verotoxigenic *Escherichia coli* and *Yersinia enterocolitica*; Gram-positive infectious bacteria such as *Listeria monocytogenes*, as well as Gram-positive toxicogenic bacteria such as *Staphylococcus aureus*, *Clostridium perfringens* and *Clostridium botulinum* (Matagaras *et al.*, 2003; Tessi *et al.*, 2002).

Coliforms, especially *E. coli*, are microorganisms of concern in almost every food product, since high counts of coliforms and presence of *E. coli* in foods usually reflect unhygienic handling during production, improper storage conditions and/or postprocess contamination. Environmental cross contamination is one of the major sources of coliforms in food systems (Keeratipibul *et al.*, 2009). On the other hand, *Salmonella* is a microorganism of paramount importance for the poultry industry, indicated by the large number of poultry products recalls due to possible or confirmed contamination with the pathogen. Animals other than humans are major reservoirs of *Salmonella*. Even when these bacteria are not a normal part of the animal's microflora, they are readily acquired from the environment and are harbored in the intestinal tract of mammals and birds. Intestinal colonization and contamination occur on farms and during transport for slaughter, and are favored by intensive rearing of animals kept together in the same facility. The sanitary

conditions of the feed supply and of the environment to which livestock and poultry are exposed influence the proportion of animals infected with *Salmonella*. Furthermore, when moving animals from the farms to the slaughterhouses there is an additional transfer of salmonellae through feces, increasing the numbers of microorganisms prior to entering the processing plants (Bryan and Doyle, 1995).

The mechanism of contamination of carcasses with *Salmonella* involves retention of bacteria in a liquid film on the skin from which they migrate into the skin and become entrapped in ridges and crevices. Moreover, this microorganism has the ability of rapidly adhering to the polysaccharide materials surrounding collagen fibers in the skin. Once they infiltrate under the skin and attach to the muscle tissue, they are inaccessible to bacteriocidal substances or processes that may be applied, posing a major risk of contaminating subsequent food processing and preparation environments (Firstenberg-Eden, 1981; Thomas and McMeekin, 1981).

2.4. *Salmonella* Pathogenesis

The primary mode of transmission of *Salmonella* is the fecal-oral route, but infection via mucous membranes of the conjunctivae or upper respiratory tract is suspected (Quinn *et al.*, 2002). Both non-specific and specific host defenses attempt to quell the infection with pathogenic organisms. After ingestion, the organism must survive the acidity of the gastric fluid. The vast majority of the bacteria perish in the stomach, but with a large inoculum, enough bacteria survive to reach the distal small intestine and colon (Chapman, 2006). One study on mice, suggests that gut luminal contents and composition may be critical to establishing infection in the intestinal epithelium (Clark *et al.*, 1998). Volatile organic acids produced by the indigenous normal anaerobic flora inhibit the growth of *Salmonella* and the normal flora usually block access to attachment sites required by the *Salmonella* species. Disruption of the normal intestinal flora by factors such as antibiotic therapy, diet and water deprivation increases the host's susceptibility to infection. Reduced peristalsis, stress due to the transportation and the overcrowding also predispose to colonization of the intestine by the *Salmonella* (Quinn *et al.*, 2002).

Although the exact mechanism of *Salmonella* invasion into intestinal epithelium is unclear, studies have documented early cellular invasion in swine using electron

microscopy (Meyerholz *et al.*, 2002). Bacteria preferentially adhered to microfold (M) cells associated with the follicle-associated epithelium (FAE) initially, but later were observed invading other cell types as well (goblet cells and enterocytes). Their study suggests that M cells are targeted early in the disease since they lack surface barriers such as glycocalyx and mucus, which present on absorptive enterocytes and goblet cells. Van Asten *et al.* (2005) reported that the bacteria were also observed migrating through crevices formed by extruded enterocytes (a process of normal cell turn over), suggesting *Salmonella* takes advantage of these sites for invasion.

There are multitudes of gene products that are required for full virulence and host interaction. In enteric bacteria, these genes are arranged into large clusters on the chromosome called *Salmonella* pathogenicity islands (SPI) so the proteins they encode can be efficiently produced at the correct time and location. *Salmonella* spp. possess five pathogenicity islands, each encoding different virulence factors that are needed for different phases of bacterial invasion. *Salmonella* pathogenicity island 1 (SPI1) is necessary for the bacterial invasion of the intestinal epithelial cells (intestinal phase), SPI 2, 3, and 4 are required for bacterial growth and survival inside the host (systemic phase), and SPI 5 mediates intestinal inflammation and fluid secretion. Finally, some virulence genes are located extra chromosomally on a highly conserved plasmid and appear to promote bacterial growth and prolonged survival in the host. *Salmonella* pathogenicity island-1 is the most studied of the five SPIs and is responsible for encoding the type III secretion system (TTS). The TTS is a series of proteins that assemble to form a “molecular syringe” to gain access to eukaryotic cells and introduce effector proteins (Chapman, 2006)

The first step to *Salmonella* invasion is the attachment of adhesion pili to the surface of intestinal mucosal cells. Differences among adhesion pillus have been identified among various serotypes (Isaacson and Kinsel, 1992). Physiologic or environmental cues cause bacteria to sequentially upregulate the components of SPI 1, including the subunits of the TTS system and complex hierarchy of effector proteins (Gruenheid and Finlay, 2003). *In vitro*, the optimal conditions for the expression of the TTS include slight alkalinity, low oxygen and high osmolarity (Lostroh and Lee, 2001). Once formed, the type III secretion system allows bacteria to create a hollow “pipeline” spanning both the bacterial and the host cell membranes. The channel created allows the bacteria to deliver effector proteins directly into the host cell cytosol and enter the cell. These proteins often resemble the

host's own proteins, thus allowing them to evade the cellular defense systems (Blaylock and Schneewind, 2005). Some of the translocated proteins initiate the signal transduction pathways and promote the actin cytoskeletal rearrangements, creating a characteristic “membrane ruffling”. Further plasma membrane manipulations create invaginations and eventually seal off around the invading microorganism (Chapman, 2006).

Once internalized, *Salmonella* have evolved mechanisms for avoiding degradation by lysosomal vesicles. The *Salmonella* containing vesicle (SCV) continues to manipulate the host cell by recruiting actin molecules to the vacuole surface. This actin coat may act as a protective barrier preventing fusion with host oxidase-containing vacuoles. Translocation of bacteria to adjacent cells can occur across the basolateral border trafficking them into deeper structures of the lamina propria. In this region, the bacteria gain access to phagocytic cells, especially tissue macrophages for the invasion and the eventual dissemination to other organs (Chapman, 2006). One study demonstrated that the spread of *Salmonella* from the intestinal mucosa to the liver and spleen is dependent on CD18-expressing macrophages and possibly the dendritic cells (Vasques-Torres *et al.*, 1999). Infected phagocytic cells may allow cell mediated immune mechanisms to contain the infection. Some speculate that infected phagocytes may play a permissive role in allowing the state of latent persistent infection (McCain and Powell, 1990; Powell *et al.*, 1994).

Survival and replication in phagocytes, and subsequent in lymph nodes, can lead to extra intestinal dissemination. Bacteria delivered to circulation are removed by cells of reticuloendothelial system, toxic effects of lipopolysaccharides (LPS) result in over stimulation of the host cytokine response, and the ultimate effects include inflammation, shock, fever and death (Songer and Post, 2005). The studies on the effect of LPS on poultry are limited compared with studies using mammals because it was generally considered that birds may be relatively more resistant to endotoxins than mammals (Roeder *et al.*, 1989). However, in recent years many studies on the effects of LPS on chickens have been reported using different *in vivo* and *in vitro* parameters. Those studies suggest that birds also show many similar patterns of response to endotoxins as mammals (Sunwoo *et al.*, 1996; Koh *et al.*, 1996; Rauber *et al.*, 2012).

2.4. Techniques Used for the Isolation and Identification of *Salmonella*

There are different methods for the detection of *Salmonella* and these methods can be

divided into: Conventional methods, Immunological techniques i.e. ELISA, and Molecular methods i.e. the polymerase chain reaction (PCR) (Van der Zee and Huis int't Veld, 2000).

2.4.1. Conventional methods

Conventional methods for *Salmonella* identification are accomplished by cultural methods. These cultural procedures generally have four distinct phases: Initial isolation (pre-enrichment in a non-selective medium, selective enrichment, isolation using selective agar media), biochemical and serological tests to confirm that those are *Salmonella* and to determine their serovar. The detection of *Salmonella* in some specific veterinary samples can also be accomplished by omitting one or more of these steps. This depends on the type of sample, the infection level and the reasons for investigation. Direct incubation in a selective broth or direct plating on selective agar can be used for clinical materials, such as feces, post-mortem organ samples (intestine, liver, spleen, gonads) and rectal and/or cloacal swabs, which are expected to contain high numbers of *Salmonella*, in contrast to those samples that generally harbor low levels of *Salmonella* spp., such as animal feed, litter, environmental swabs, egg and egg materials, require all four phases of the cultural procedure to be performed (Van der Zee and Huis int't Veld, 2000).

2.4.1.1. Pre-enrichment in non-selective medium

Pre-enrichment in non-selective medium allows resuscitation of any injured cells and multiplication of the target organism and others present in the sample (Van der Zee and Huis int't Veld, 2000). The early studies showed that the direct plating and the direct selective enrichment of certain types of samples were often unsuccessful for the detection of *Salmonella*. Typically, samples that were dried, heated, irradiated or otherwise processed required the use of non-selective pre-enrichment for the optimal recovery of *Salmonella*. In these samples, *Salmonella* may be present but is damaged or sub lethally injured. These organisms, although still viable and able to cause disease under right conditions, are easily killed if placed into harsh environment of the most selective-enrichment broths, especially when incubated at higher temperatures (Waltman, 2000). Several pre-enrichment media have been advocated. Lactose broth (LB) was perhaps the first to receive wide spread use, but there has been concern that LB, because of the fermentation of lactose and resulting acidity, would allow the pH to fall to a level that is

inhibitory or lethal to *Salmonella* (Hilker, 1975). Other pre-enrichment media has been formulated without a fermentable sugar and with greater buffering capacities, e.g. buffered peptone water (BPW) (Waltman *et al.*, 1995). BPW is the medium of choice for the majority of food products according to ISO-6579 (Anon, 2002). For many years, pre-enrichment of these samples was not advocated, because it was thought that non-selective enrichment would allow over-growth of organisms other than *Salmonella* and give false-negative results (Waltman, 2000). The incubation of pre-enrichment for 18-24 hours is recommended to allow resuscitation of *Salmonella* before the transfer into the selective-enrichment media and the recommended incubation temperature for pre-enrichment is 35-37°C (Waltman *et al.*, 1993).

2.4.1.2. Enrichment in selective medium

This method is to allow the survival or the growth of *Salmonella*, while inhibiting other accompanying organisms in the selective broth (Van der Zee and Huis in't Veld, 2000). Typically, in chronic infections, carrier animals or environmental samples, the numbers of *Salmonella* are low, especially relative to the high numbers of the other bacteria. These samples should be inoculated into the selective enrichment media for the optimal recovery of *Salmonella* (Waltman *et al.*, 2002).

Various enrichment techniques have been described including prolonged enrichment (extended sample incubation times), secondary enrichment (using two or more enrichments in series) and delayed enrichment (storage of enrichment samples at room temperature for a period of time). Delayed secondary enrichment involves a primary incubation in enrichment broth, with incubation at 37°C or 42°C (depending on the media) for 24 hours followed by further incubation at room temperature for 3-5 days. This is followed by the subculturing into new enrichment broth and a second incubation at 37°C for 24 hours (Waltman, 2000).

The mechanism responsible for the improvements in *Salmonella* recovery with delayed secondary enrichment is unknown; however, some speculated that the prolonged incubation at the room temperature may favor the growth of the species in the lowest numbers (Nietfeld *et al.*, 1998). Bacteria in large numbers reach stationary phase faster and then make up a larger proportion of dying cells, while the bacteria in lower numbers (e.g.

Salmonella) have not yet reached stationary phase (Nietfeld *et al.*, 1998). There are currently three major types of selective enrichment media for *Salmonella*.

Tetrathionate broth (TTB): It enriches *Salmonella*, including *S. typhi* and sometimes *Shigellae*, but permits the growth of *Proteus* spp., which may reduce the tetrathionate and thus impair the selectivity for *Salmonella*. There are several formulations of the selective enrichments based on tetrathionate (TT) (Old, 1996). They include Mueller-Kauffmann (MKTT) as formulated by Mueller and modified by Kauffmann, who added the brilliant green dyes and bile salts as selective agents (Van Der zee, 2003). The addition of 0.001% of brilliant green dyes inhibits the growth of *Proteus* spp., but also makes it rather too inhibitory to *S. typhi* and *Shigellae* and/or addition of novobiocin as an alternative to brilliant green (Old, 1996). The addition of 40mg novobiocin per liter of medium before the addition of iodine serves to overcome the interference by *Proteus* (Jeffries, 1959).

Selenite broth (SB): Several modifications to the original selenite-based medium as described by Leifson in 1936 have been proposed. Well known modifications are selenite F (SF), selenite cystine (SC), dolcitol selenite and selenite brilliant green (SBG) (Fricker, 1987). SF broth is probably the most used enrichment medium for specimens that may contain either *Salmonella* or *Shigellae*, which is excellent for *S. typhi* and *S. dublin* but some species such as *paratyphi* A and *choleraesuis* and some *Shigella* spp. may fail to multiply (Old, 1996).

Rappaport-Vassiliadis (RV) enrichment broth: The original Rapapport medium contained malachite green and magnesium chloride as selective agents (Rapapport *et al.*, 1956), and was modified by Vassiliadis (Vassiliadis *et al.*, 1970). This medium has become known as RV medium and is now widely used in Europe, and has been reported to be more efficient than the other enrichment media for isolation of *Salmonellae* from feces, water and foodstuffs and more positive result can be obtained if incubated for 48 hrs (Fricker, 1987).

The original formulation described by Rappaport *et al.* (1956) was specifically developed to exploit the four characteristics of *Salmonella* species when compared with other Enterobacteriaceae.

1. The ability to survive at relatively high osmotic pressures.

2. To multiply at relatively low pH values.
3. To be relatively more resistant to malachite green.
4. To have relatively less demanding nutritional requirements

Several studies have mentioned that RV enrichment was better than either TTB or SB (Bager and Peterson, 1991; Oboegbulem, 1993; Schlundt and Munch, 1993; Waltman *et al.*, 1995). Hammack *et al.* (1999) recommended that for the recovery of *Salmonella* spp. from food with a low microbial load, TTB at 35°C and RV at 42°C can be used. In a comparative study, Waltman *et al.* (1995) compared SF, SC and SBG with TTB and RV enrichment broth. The TTB and RV enrichment results were better than those from the use of selenite enrichments.

2.5.1.3. Isolation in selective medium

Various plating media have been developed for the isolation of *Salmonella* by using the principles of selectivity and differentiation. Selectivity involves the incorporation of inhibitory substances into the media that selectively inhibit other bacteria. The differential characteristics of a plating media involve the addition of substances that differentiate *Salmonella* colonies from other bacteria (Waltman, 2000).

Strategies employed by selective media include inhibitor substances (such as metals, chemicals and antibiotics), which hinder the growth of undesirable bacteria, buffers that optimize the pH for certain microorganisms, supplements (vitamins, blood, serum etc) that enhance the growth of fastidious bacteria, selective carbohydrates (lactose, succors, maltose, dextrose, xylose) as energy sources, proteins (peptone, casein, typtones) for metabolism and indicator substances (Chapman, 2006).

Many different selective media are available for the isolation of *Salmonella* including Bismuth Sulphite agar (BS), Deoxycholate Citrate agar (DCA), Brilliant Green agar (BG), Hektoen Enteric agar (HE), Manitol Lysine Crystal Violet Brilliant Green (MLCVBG) agar, Modified lysine Iron (MLI) agar, Novobiocin Brilliant Green Glycerol Lactose (NBGL) agar, Salmonella-Shigella agar (SS), Xylose Lysine Deoxycholate agar (XLD), and Xylose Lysine Tergitol 4 (XLT4) agar (Waltman, 2000). The majority of *Salmonella* are non-lactose fermenters and produce pale colonies on MacConkey (MAC) agar. On XLD medium, the majorities of *Salmonella* serotypes produce hydrogen sulphide and have

red colonies with black centers (caused by H₂S production) (Quinn *et al.*, 2002). On the HE medium, colonies are blue-green or blue, with or without black centres (Songer and Post, 2005). On BS medium, typical *Salmonella* colonies appear black with a characteristic black sheen. On DCA, their colonies are clear with black centers and on BG medium their colonies appear pink to red with a reddening of the media (Waltman, 2000). On modified lysine iron (MLI) agar, the typical *Salmonella* are blue with black centers. On SS medium, typical *Salmonella* are colorless with black centers and on RA, the differential characteristics involve the fermentation of propylene glycol, typical *Salmonella* appearing as crimson-red colonies (Waltman, 2000).

Since 1990, several chromogenic media have been developed to detect *Salmonella* such as Rambach agar (RA) (Rambach, 1990), *Salmonella* detection and identification agar (SM-ID) (Dusch and Altwegg, 1995), Chrom agar (Gaillot *et al.*, 1999), α and β -II Chromogenic Medium (ABC) (Perry *et al.*, 1999), Chromogenic *Salmonella* esterase agar (Cooke *et al.*, 1999) and Rainbow *Salmonella* agar (Manafi, 2000). They are based on biochemical characteristics and are highly specific (Rall *et al.*, 2005). Incorporation of chromogenic enzyme substrates into their formulations for the detection of *Salmonella* allows to bacterial enzymes, such as β -galactosidase in the case of *Salmonella*, to convert the chromogenic substrate and to produce a color change in the colony. With ABC medium (α and β -chromogenic), which incorporates two chromogenic substrates, the first substrate, 3,4-cyclohexenoesucletin- β -D-galactoside (CHEGAL), is incorporated to visualize β -galactosidase-producing organisms i.e. Enterobacteriaceae as black colonies in the presence of iron, and the second substrate, 5-bromo-4-chloro-3-indolyl- α -D-galactopyranoside (X-a-Gal), is hydrolyzed by strains of *Salmonella* spp., which is visualized as green colonies (Perry *et al.*, 1999). Incorporation of chromogenic substrates into selective agars has a significant impact on cost. However, it is believed that this is offset by the substantially reduced labor time and consumable costs associated with the detection of *Salmonella* spp. on traditional agars (Perry *et al.*, 1999).

It is clear that no single medium will reliably detect all known *Salmonella* serotypes. For this reason it is recommended by various regulatory bodies that a combination of complementary media be used for the reliable detection of all *Salmonella* spp. (Warburton *et al.*, 1994).

2.5.1.4. Biological and serological confirmation

Further confirmation of *Salmonella* is accomplished by using series biochemical tests. The more biochemical tests that are performed on a suspect colony, the more sensitive the results (Chapman, 2006).

Kligler's iron (KI) agar and triple sugar iron (TSI) agar establish the ability of the organism to ferment different carbohydrates. These agars contain glucose and lactose in a ratio of 1:10. Non carbohydrate-fomenters fail to convert either glucose or lactose to acid so the media, which contains phenol red as a pH indicator, remains unchanged. Bacteria which ferment glucose, but not lactose, will initially produce acid as a result of glucose fermentation. However when the glucose supply is exhausted, these organisms will utilize amino acids in the aerobic portion of the media, and revert the media to the alkaline color of red. Organisms that are capable of fermenting both glucose and lactose will result in acid production (and a yellow color change) in both the superficial and deep portions of the agar. Sodium thiosulfate and ferrous sulphate are added for the detection of hydrogen sulphide production. Additional testing include the ability of bacteria to use lysine as a sole carbon source in lysine iron (LI) agar, The medium contain a small amount of glucose as an energy source and once this is exhausted, the decarboxylase enzyme is activated to utilize the amino acids present in the media. Decarboxylation of lysine, or removing a molecule of CO₂, produces a pH shift in the medium as the alkaline amines are formed. Bromcresol purple is the indicator substance which confirms alkalinization (Chapman, 2006).

Further differential testing may be accomplished by examining for the presence of bacterial urease, citrate utilization, indole, methyl red and Voges Proskauer. These tests are followed by serological agglutination, then serovar and phage group identification (Songer and Post, 2005).

For confirmation of the biochemical tests, multitest strip identifications systems are available to confirm the identification of the Enterobacteriaceae. Some of these systems require overnight incubation as such in API 20E (Biomerieux, UK) while others provide rapid results in as fast as 4 hours as such in RapIDTM one system, Remel, (USA) (Chapman, 2006).

The laboratory procedures for detection and identification of *Salmonella* by conventional methods are laborious and time consuming taking 3-5 days (Kumar *et al.*, 2008). Various rapid methods for the detection of foodborne *Salmonella* have been reported such as chromogenic media, bacterial agglutination, fluorescent antibody technique, enzyme linked immunosorbent assay (ELISA), immunomagnetic assays (Brigmon *et al.*, 1992; D'Souza and Jatkus, 2003), PCR (Oliveira *et al.*, 2002; Whyte *et al.*, 2002) and a complement fixation test (Quinn *et al.*, 2002).

2.5.2 Immunological methods

More rapid immunological screening methods of detection have been devised to detect *salmonella* such as rapid agglutination assay, Enzyme-linked immunosorbent assay (ELISA) and lateral flow immunoassays.

Enzyme-linked immune sorbent assay (ELISA): also known as an enzyme immunoassay (EIA), is a biochemical technique used to detect the presence of an antibody or an antigen in a sample. It has been widely used for *Salmonella* detection because they allow the sensitive and specific detection (Smith *et al.*, 1995).

Immunoassays offer the ability to detect and distinguish serovars of *Salmonella*. However, immunoassays are hard to incorporate into a group format for multiple targets because high level of cross-reactivity between antibodies limits the number of targets that can be detected on the same group. Use of immunomagnetic beads (IMBs) in detection assays has been reported, and it shows great potential in *Salmonella* detection from complex matrices or environmental samples (Gehring *et al.*, 1996).

2.5.3 Molecular methods

The breakthrough on DNA amplification theory and subsequent discovery of highly efficient and thermal-tolerant DNA polymerase enable the emergence and evolution of molecular-based pathogen detection assay. Methods such as PCR and real-time PCR are widely used in detection, diagnosis, genetic characterization, and other biological research area, because of their high speed, sensitivity, specificity and reproducibility. PCR can exponentially generate thousands to millions of copies of a particular DNA sequence in vitro across several orders of magnitude (Mullis *et al.*, 1986).

Polymerase chain reaction: PCR has emerged as a rapid and sensitive diagnostic tool for the detection of small quantities of target DNA. Many *Enterobacteriaceae* share common virulence genes and phylogenetic similarities, therefore a target gene must be genus-specific for *Salmonella*, and not shared by closely related bacteria (Chapman, 2006). Many oligonucleotide primer sets have been describe for the detection of *Salmonella*, including those associated with the invasion genes *invA* (Rahn *et al.*, 1992; Stone *et al.*, 1994) and *invE* (Stone *et al.*, 1994), histidine transport operon *hisJ* (Cohen *et al.*, 1993), SPI1 invasion gene *hilA* (Guo *et al.*, 2000), virulence plasmid gene *spv* (Gulig *et al.*, 1993). Recent studies describe a serotype-specific PCR method of differentiating *S. pullorum* from other serotypes using the *rfbS* gene (Desai *et al.*, 2005).

Real time PCR: A number of real-time PCR based assays for the detection of *Salmonella* have been developed and published in recent years (Moore and Feist, 2007). Real time PCR technology has improved the accuracy of nucleic acid amplification compared to the standard PCR assay. By using a dual labeled internal fluorogenic probe, which acts as a molecular beacon (Chen *et al.*, 2000). Real-time PCR uses an internal fluorogenic probe that when bound to the target sequence is detected as a fluorescent signal during the reaction process, so the need for the post reaction handling is eliminated, in comparing to the standard or classical one (Chapman, 2006).

The culture and PCR methods used for the detection of *Salmonella* from clinical fecal samples are of similar sensitivity. However, using culture, results are available within 2-3 days, whereas those obtained by the real-time PCR assays could be available within 3 hours, which can be advantageous for the rapid intervention and the appropriate treatment (Amar *et al.*, 2004).

2.6. Prevention and Control of *Salmonella*

Prevention and control measures should be designed based on the two distinct categories, *Salmonella* infections that have a direct negative impact on the poultry population, and *Salmonella* infections of importance to public health (Breytenbach, 2004).

Prevention of Salmonellosis by the implementation of hygiene measures is difficult and use of antibiotics may give rise to the emergence of resistance problems (Mastroeni and Menager, 2003). Reducing *Salmonella* prevalence requires a multi-hurdle approach at

all stages of breeding, hatching, grow-out, transportation and processing. No silver bullets can be added at a single point in production or processing that will completely eliminate *Salmonella* on chickens. Therefore, some researchers describe “competitive exclusion” as the most effective and harmless method to control *Salmonella* in poultry (Ragione and Woodward, 2003, Schneitz, 2005). Attenuated DNA recombinant live *Salmonella* vaccines combined with comprehensive control strategy in animals, feed and animal food stuffs with restrictions on the infected flocks until they have been cleaned up from infections and mandatory testing before slaughter like that being implemented in Sweden will help reduce salmonellosis (Kang *et al.*, 2002; Boqvist and Vagsholm, 2005). The prevention of paratyphoid *Salmonella* infection which has greater public health consequences requires a comprehensive control strategy including regular monitoring, strict biosecurity, sourcing feed containing no animal protein, and vaccination (Breytenbach, 2004).

Ensuring safe food production requires knowledge on the nature and origin of the animals, animal feed, the health status animals at the farm, the use of veterinary medicinal products, the results of any analysis of the samples taken at the farm and slaughter data regarding ante-mortem and post-mortem findings and the risks associated with post-harvest production stages (Snijders and Knapen, 2002), or no part of the food chain can be regarded alone but has to be seen as part of the whole. A holistic approach to fresh meat storage and retailing must therefore start with the living animal and cannot end with the sale of the meat. It must also include the consumers (Nowak *et al.*, 2006). Additional measures to control secondary contamination could be prevention of contamination by cleaning and disinfection, hygiene of personnel and proper processing (Sinell, 1995). Growth of microorganisms in meat and poultry products can be controlled by maintaining a cold chain at 10°C, especially for *Salmonella* during transport and storage (Coleman *et al.*, 2003).

Inactivation of potential microbiological pathogens in the end-product may appear attractive, as it reduces the spoilage micro-organisms too (Farkas, 1998); however, other options such as antimicrobial packaging (Quintavalla and Vicini, 2002), vacuum packaging, intelligent packaging and smart active labeling with freshness and spoilage indicators might prove better choices for consumer health protection (Nowak *et al.*, 2006). Provided the initial production stages have low bacteria and/or are pathogen free, the approach to preventing spoilage of fresh meat is to keep the products at low temperatures,

where in most countries the storage temperature is prescribed by law. Enabling rapid identification of microbial contamination to allow rapid response (Doyle and Erickson, 2006), knowledge and attitude of the consumers (Woteki *et al.*, 2001), personal hygiene of food handlers (Nowak *et al.*, 2006), consumer perception of food safety (Redmond and Griffith, 2004) and continuous further education (Fischer *et al.*, 2006) are equally important to achieve food safety practices.

2.7. Antibiotic Resistance of *Salmonella*

Because of the extensive use of antimicrobial agents in animals and human therapy resistance became a public health concern (Sefton, 2002). Drug resistant *Salmonella* emerges in response to antimicrobial usage in humans and in food animals and the selective pressure from the misuse of antimicrobials is a major driving force behind the emergence of resistance (WHO, 2013). A recent estimate in the United States suggests that 24.6 million pounds of antibiotics are given to animals each year as growth promoters at sub-therapeutic amounts in their feed compared to 3 million pounds consumed by humans (White *et al.*, 2001).

The development of resistance in *Salmonella* toward antimicrobial agents is attributed to multiple mechanisms, including adaptation by the organism of an alternative metabolic pathway to bypass the inhibited reaction, production of enzymes that inactivate antimicrobial agents, through degradation or structural modification, reduction of bacterial cell permeability to antibiotics, activation of efflux pumps and modification of cellular targets for drugs (Sefton, 2002; Carter and Wise, 2004).

An efficient route of acquisition and dissemination of resistance determinants is through mobile genetic elements such as plasmids factors, transposons or integrons that harbor specific resistance genes (Fluit and Schmitz, 1999). These mobile genetic elements can spread horizontally among the bacteria of the same serovars, subspecies, or species, but also among those of different species or even different genera (Thomas and Nielsen, 2005). Among important resistance determinants, complex integrons have been implicated, which occur either on plasmids or integrated into the bacterial chromosome (Goldstein *et al.*, 2001). However, most integrons are contained within chromosomes, and class 1 integrons are one of the single biggest contributors to multidrug resistant infections,

carrying resistance to many antibiotics in diverse pathogens on a global scale (Stokes *et al.*, 2006). Resistant genes carried by integrons can be transferred horizontally to the other bacteria via conjugative plasmids and transposons (Hamada *et al.*, 2003).

One of the studies in Spain reported high percentages of resistance of *Salmonella* isolates to sulfadiazine, neomycin, tetracycline and streptomycin, which might be the result of the use of antibiotics as a prophylaxis, growth promoter or treatment (Carraminana *et al.*, 2004). In another study in Spain, 55 *S. abortusovis* strains isolated from Spanish ovine flocks were screened for resistance to 18 antimicrobial agents and reported that all strains showed full streptomycin and spectinomycin resistance. Although 23 out of 55 strains were sulphamethoxazole resistant, only four of them also expressed resistance to trimethoprim/sulphamethoxazole (Valdezate *et al.*, 2007).

A study in Alberta, Canada indicated a high resistance of *Salmonella* isolates from food and food animals to ampicillin, streptomycin, sulfamethoxazole and tetracycline (Johnson *et al.*, 2005). Over the past decade in Nepal, increasing antibiotic resistance in *S. enterica* has led to a shift in the antibiotics used against this organism from chloramphenicol and ampicillin to trimethoprim/sulfamethoxazole, fluoroquinolones and ceftriaxone, where only a 16-40% positive response to treatment has been achieved (Pokharel *et al.*, 2006). In another study, of 380 *Salmonella* isolates from animal origin in the US, 82% of the isolates were resistant to at least one antimicrobial, and 70% to three or more antimicrobials. Resistance was most often observed to tetracycline, followed by streptomycin, sulfamethoxazole, ampicillin, chloramphenicol, kanamycin, amoxicillin/clavulanic acid, and ceftiofur (Zhao *et al.*, 2007).

In USA, Kalchayanand *et al.* (2007) reported that 174 isolates from lamb carcasses were susceptible to all antibiotics tested, with only one isolate, from a pre-evisceration carcass, being multidrug-resistant (MDR) *Salmonella*. The MDR *Salmonella* was the serotype *typhimurium* and was resistant to amoxicillin-clavulonic acid, ampicillin, chloramphenicol, streptomycin, sulfisoxazole, and tetracycline.

In Van, Turkey, Aksakal *et al.* (2009) found that 9 *S. saintpaul* strains isolated from sheep were susceptible to ampicillin, enrofloxacin, tetracycline, oxytetracycline, gentamicin, neomycin, chloramphenicol, spectinomycin, cephalothin, amoxicillin/clavulanic acid, trimethoprim/sulfamethoxazole and nalidixic acid and they

were resistant to erythromycin and novobiocin, and two of the strains from rectal isolates were resistant to penicillin G. However Davies *et al.* (2004) in Great Britain reported that *Salmonella* strains isolated from feces of animals slaughtered in abattoirs were sensitive to amoxicillin/clavulanic acid, ampicillin, chloramphenicol, gentamicin, neomycin, trimethoprim/sulfamethoxazole and nalidixic acid. Molla *et al.* (2006) reported that *Salmonella* strains isolated from feces of sheep slaughtered in abattoirs in Ethiopia were susceptible to gentamicin, neomycin and nalidixic acid, whereas they were resistant to amoxicillin/clavulanic acid, ampicillin, cephalothin, chloramphenicol, trimethoprim/sulfamethoxazole, spectinomycin and tetracycline. Intorre *et al.* (2005) in Italy found that *Salmonella* strains isolated from sheep were resistant to penicillin and erythromycin.

In Iraq, Hadad and Jemel (1990) tested 122 *Salmonella* isolates of animal origin for antimicrobial susceptibility, only 15.6% of the isolates were sensitive to all of the antibiotics used, while 84.4% of the isolates were resistant to one or more of the antibiotics tested, most frequently they were resistant to sulphafurazol, tetracycline, streptomycin and chloramphenicol, however all isolates were sensitive to ampicillin, carbnicillin, gentamycin, furazolidone, cephaloridine and nalidixic acid. Similarly, Hadad and Ali (1995) in Mosul, Iraq, reported that 72 *Salmonella* strains isolated from dairy products were resistant to tetracycline and sulphamethizole, while all isolates were sensitive to co-trimoxazole. Fifty-two isolates were resistant to three or more antibiotics and included resistant to tetracycline, sulphamethizole and streptomycin. Al-Abidy and Hadad (2007) in Mosul, Iraq, revealed that 35 *Salmonella* strains isolated from frozen poultry products were totally resistant to erythromycin and 32 isolates were resistant to 4 antibiotics (gentamicin, tetracycline, amoxicillin and nalidixic acid). Six patterns of multiple antibiotic resistant were found the most frequent resistance was to gentamicin, erythromycin, amoxicillin, tetracycline and nalidixic acid.

3. MATERIALS AND METHODS

3.1. Materials

A total of 100 chicken carcasses were collected from the local markets and butcheries during June 2013 to January 2014 in the Northern Iraq. The meat samples used in the study were chosen either fresh or frozen (Table 3.1).

Table 3.1. Collection areas of chicken samples

Source	Fresh	Frozen	No. of Sample
Duhok	25	30	55
Arbil	25	20	45
Total	50	50	100

Buffered peptone water, Rappaport-Vassiliadis (RV) enrichment broth, ABC chromogenic agar, Xylose lysine deoxycholate (XLD) agar, nutrient agar, Kligler iron (KI) agar, Simmons citrate (SC) agar, Brilliant green (BG) agar, Urease broth, Mueller Hinton (MH) agar and Brain heart infusion (BHI) broth were purchased from LabM, UK. Antibiotic discs used in the study were purchased from Bioanalyse, Turkey (Table 3.2.). RapID™ ONE was purchased from Remel Inc (USA). *Salmonella* somatic (O) and flagellar (H) antisera were obtained from Plasmatec, UK.

Table 3.2. Antibiotics used in the study

	Antibiotic	Abbreviation	Concentration (□g)
1	Amoxicillin/Clavulanic	AM	30
2	Carbenicillin	CT	25
3	Chloramphenicol	C	10
4	Ciprofloxacin	CIP	10
5	Doxycycline	DOC	10
6	Enrofloxacin	ENR	15
7	Erythromycin	E	15
8	Gentamicin	GN	30
9	Kanamycin	K	30
10	Nalidixic acid	NA	30
11	Nitrofurantoin	F	30
12	Tetracycline	TE	30
13	Trimethoprim	TMP	10

3.2. Sampling of Chicken Meats

Fresh carcasses were broken into pieces under hygienic conditions in the laboratory. The pieces from neck, wings, breast and drumstick were placed in sterile sample-collection bags, properly labeled and stored in a refrigerator or subjected to immediate analysis.

Frozen carcasses stored at freezer temperature for no longer than a week were thawed at the room temperature and broken into pieces under hygienic conditions in the laboratory. The pieces from neck, wings, breast and drumstick were placed in sterile sample-collection bags, properly labeled and stored in a refrigerator or subjected to immediate analysis.

3.3. Isolation

Representative meat samples were taken from neck, wings, breast and drumstick and grounded. Twenty-five grams of the meat sample were homogenized in 225 mL of peptone water using a stomacher (Bagmixer, Interscience) and incubated aerobically at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 18 ± 2 hours. A volume of 0.1 mL homogenate was added into 10 mL of RV broth, vortexed and incubated aerobically at $41.5^{\circ}\text{C} \pm 2$ for 24 ± 3 hours for the selection of *Salmonella*. One loop of the broth culture was plated on a XLD agar and one loop on an ABC agar for the subcultivated of *Salmonella*, and both were incubated aerobically at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 24 ± 3 hours. Three suspected colonies having red color with black centers on the XLD agar and green color on the ABC agar were picked up and transferred separately on nutrient agar plates and streaked for increasing the number of *Salmonella*. Nutrient agar plates were incubated at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 24 ± 3 hours. The colonies grown on the nutrient agar plates were later taken into biochemical and serological confirmation tests.

3.4. Biochemical Confirmation

The following biochemical tests were carried out on the suspected colonies according to the methods described by Quinn *et al.* (2002); Brown (2004).

3.4.1. Glucose and lactose fermentation and hydrogen sulfide production in Kligler iron agar

It was applied for differentiation of microorganisms on the basis of glucose and lactose fermentation and hydrogen sulfide production (H_2S). Three-four colonies from the nutrient agar plates were stabbed into the butt and streaked the entire slant of a KI agar tube by using the sterile loop and incubated at 37°C for 18-24 hours. Typical *Salmonella* colonies in the KI agar tube were observed to have alkaline (red) slants and acid (yellow) butts (indicating only glucose fermentation; glucose-positive), to form bubbles from glucose fermentation and black precipitates from the hydrogen sulfide formation. However, the agar slant was yellow when *Salmonella* isolated fermented lactose (lactose-positive). Thus, the suspected *Salmonella* colonies were subjected to further tests for the confirmation.

3.4.2. Citrate utilization in Simmons citrate agar

It was applied to detect the ability of a microorganism to utilize the citrate as the sole carbon and energy source for growth and an ammonium salt as the sole source of nitrogen. A slant of SC agar tube was inoculated by 3-4 suspected *Salmonella* colonies isolated from the nutrient agar plates, and incubated at 37°C for 18-24 hours. Positive reactions were indicated by the growth of *Salmonella* on the SC agar tubes with the change in the color of agar from green to intense blue.

3.4.3. Urea utilization in Urease broth

This test is used to determine the ability of a microorganism to hydrolyze urea to form ammonia by action of the urease enzyme. *Salmonella* is always urease-negative. The suspected *Salmonella* colonies (3-4 colonies) from the nutrient agar were incubated on a urease broth tube at 37°C for 18-24 hours. While a urease-positive culture produced an alkaline reaction in the medium, evidenced by pinkish-red colour of the medium, urease-negative organisms did not change the color of the medium, which was pale yellow-pink.

3.4.4. RapID™ ONE test

The suspected *Salmonella* colonies (3-4 colonies) from the nutrient agar plates were subcultured on a BG agar and incubated at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 24 ± 3 hours. After incubation, 3-4 colonies were picked up and transferred into 2 mL of the RapID™ ONE inoculation fluid (6 g KCl, 0.5 g CaCl_2 , 1000 mL demineralized water) to achieve an inoculum with visual turbidity equal to a #2 McFarland turbidity standard. The suspension was vortexed and transferred into the upper right-hand corner of the panel of RapID™ ONE system by using pipette. The panel was backed away from the reaction cavities at a 45° angle and racked gently side to side to evenly distribute the inoculum along rear baffles. The panel was slowly tilted forward toward the reaction cavities until the inoculum follows along the baffles into the reaction cavities. The inoculated panel was incubated at 37°C for 4 hours. After 4 hours incubation, the lid over of the reaction cavities was pulled off and then 2 drops of RapID™ ONE reagent was added to cavities 15 (PRO) through 17 (PYR). Then cavities 1 (URE) through 18 (ADON) were scored and recorded in the report form by using test codes. Then 2 drops of RapID™ ONE spot indole reagent was added to cavity 18 (ADON/IND), and was allowed for 10 seconds for color development and the score of cavity 18 (IND) was recorded in the report form by using the test code. The code numbers for (+) reaction in each triplet of biochemical reactions were added together. This gave a 7-digit (profile number) for the bacterium. The genus name was obtained by entering the 7-digit profile number into the computerized program (software) provided with RapID™ ONE kit.

3.5. Serological Confirmation

Three separate drops of normal saline (0.85% sodium chloride) were placed on a clean glass slide. The suspected *Salmonella* colonies from the nutrient agar were added on two saline drops and mixed thoroughly on the slide to obtain a smooth suspension. The third drop on the slide was used as a control. One drop of somatic (O) and flagellar (H) antisera was added separately on to two drops of bacterial suspension on the slide in order to obtain a manner that each isolate was tested for agglutination against both antisera at the same time. Both antisera were mixed with bacterial suspensions by sterile loops. Then the slide was gently tilted back and forth for one minute. During this period agglutinations were observed under a normal lightening condition against a black background. All

positive isolates were subcultured onto the slants of KI agar, and submitted to the Central Health laboratory, Ministry of Health, Baghdad for serotyping.

3.6. Antibiotic Sensitivity Test

Kirby-Bauer disc diffusion method (Bauer *et al.*, 1966) was used to determine the *in vitro* antibiotic susceptibility of the identified *Salmonella* isolates to various antibiotics given in Table 3.2. Antimicrobial susceptibility screening was conducted using a panel of 13 agents. The concentrations of the antibiotics tested were 10-30 µg. A standardized suspension of the isolated *Salmonella* from the KI agar was adjusted to a 0.5 MacFarland turbidity standards and diluted 1:10 for the dilution method. A sterile swab was dipped into the standardized inoculum and used to inoculate evenly the surface of already prepared MH agar. The agar was left for 15 minutes for the surface moisture to dry. A multichannel disc dispenser (Oxoid, Basingstoke, UK) was used to deposit the antibiotics discs onto the surface of the inoculated medium. The plate was then incubated at 37°C for 24 h. The zones of growth inhibition were measured with slipping calipers. The diameter was read across the center of the discs and the result were interpreted according to the manufactures instructions.

4. RESULTS

The study was designed with the estimation of chicken meat prevalence of *Salmonella* as a central goal. One-hundred fresh or frozen chicken meats from local markets and butcheries in Northern Iraq were sampled in order to provide a reasonably precise and unbiased estimate of *Salmonella* prevalence.

4.1. Isolation of *Salmonella*

Suspected *Salmonella* spp. were observed as red colonies with a black center on the selective XLD and green color on ABC agars as seen in Figure 4.1. Eleven out of 100 meat samples were found to have susceptible *Salmonella* spp. The fresh meat samples (9) had higher prevalence of *Salmonella* spp. than the frozen meat samples (2). Arbil (15.6%) was observed to have 2 times more prevalence of *Salmonella* spp. than Duhok (7.3%). The prevalence of *Salmonella* in chicken meats is given in Table 4.1.

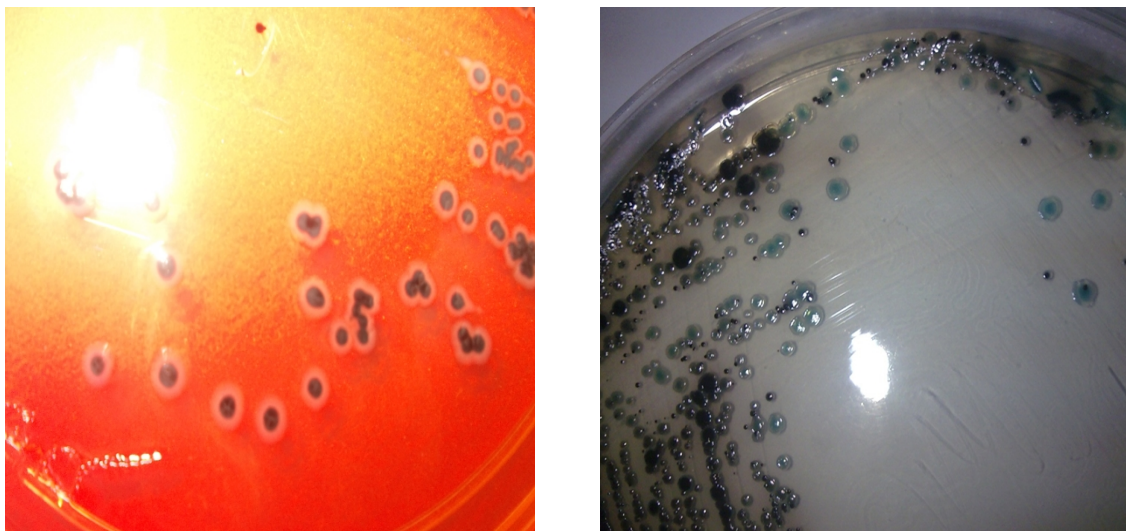


Figure 4.1. Suspected *Salmonella* spp. on the XLD (left picture) and ABC agars (right picture)

Table 4.1. The distribution of susceptible *Salmonella* spp. on chicken meat samples detected on XLD and ABC agars

Area	Sample	No. of samples	Susceptible <i>Salmonella</i> spp. Plates	
			Number	%
Duhok	Fresh	25	3	12.0
	Frozen	30	1	3.3
Arbil	Fresh	25	6	24.0
	Frozen	20	1	5.0
Total		100	11	11.0

4.2. Biochemical Confirmation of *Salmonella* with Conventional Methods

The eleven suspected agar plates were subjected to the confirmation using the KI agar, SC agar and urease broth. Typical *Salmonella* cultures in the KI agar tubes were shown to form red (alkali) slant, yellow (acid) butt, black precipitates and gas bubbles. This indicated that *Salmonella* spp. fermented glucose but not lactose and reduced sulfur compounds to H₂S. However, in some KI agar tubes, yellow (acid) slants were also observed, indicating fermentation of lactose by some *Salmonella* spp. In the SC agar tube, the color turned from green to intense blue in the presence of *Salmonella* spp. Since *Salmonella* is urease-negative, no color changed occurred in the urease broth tubes. The number of the *Salmonella* suspected tubes was 8 out of 11 suspected colonies (Table 4.2). Six of 9 fresh meat samples and all frozen meat sample (2) were found to be infected with *Salmonella* spp. after the confirmation tests. The prevalence of *Salmonella* in the meat samples was more common in Arbil than in Duhok.

Table 4.2. The distribution of *Salmonella* spp. on chicken meats confirmed on KI broth, SC agar and Urease broth

Area	Sample	No. of samples	Susceptible <i>Salmonella</i> spp.	
			Number	%
Duhok	Fresh	3	2	66.7
	Frozen	1	1	100.0
Arbil	Fresh	6	4	66.7
	Frozen	1	1	100.0
Total		11	8	72.7

4.3. Biochemical Confirmation of *Salmonella* with RapID™ ONE System

The suspected 11 agar plates were later subjected to the RapID™ ONE test for the confirmation by the conventional methods. The change in the color of the baffles was observed after the suspected *Salmonella* colonies were incubated (Figure 4.2).



Figure 4.2. Confirmation of *Salmonella* on the meat samples using RapID™ ONE test

All plates were suspected to contain *Salmonella*, indicating that RapID™ ONE test is more sensitive than the conventional methods for the confirmation of *Salmonella* ssp. The results of the RapID™ ONE test are given in Table 4.5. Two different results were obtained from the RapID™ ONE test: (1) Positive results were found in the ODC, LDC, TET and SBL baffles, the seven-digit number obtained was 4320000, and the data analysis program said 93% *Salmonella* ssp. (2) Positive results were found in the ADH, ODC, LDC, SBL and GGT baffles, the seven-digit number obtained was 6120010, and the data analysis program said 100% *Salmonella* ssp.

Table 4.3. The result of the RapID™ ONE system

No	Test code	Reactive ingredient	Results	
1	URE	Urea	-	-
2	ADH	Arginine	-	+
3	ODC	Ornithine	+	+
4	LDC	Lysine	+	+
5	TET	Aliphatic thiol	+	-
6	LIP	Fatty acid ester	-	-
7	KSF	Sugar aldehyde	-	-
8	SBL	Sorbitol	+	+
9	GUR	nitrophenyle-□, D glucuronide	-	-
10	ONPG	nitrophenyl-□, D-galactoside	-	-
11	□GLU	nitrophenyl-□, D-glucoside	-	-
12	□XYL	nitrophenyl-□, D-xyloside	-	-
13	NAG	nitrophenyl-n-acetyl-□, D-glucosaminide	-	-
14	MAL	Malonate	-	-
15	PRO	Proline-□-naphthylamide	-	-
16	GGT	Glutamyl-□-naphthylamide	-	+
17	PYR	Pyrrolidonyl-□-naphthylamide	-	-
18	IND	Tryptophane	-	-

4.4. Serological Confirmation of *Salmonella*

The results of the serological test showed that the isolates gave positive reactions (agglutination) with polyvalent O and H antisera as shown in Figure 4.3. The suspected 11 cultures were serotyped by the Central Health Laboratory in Baghdad and identified as *S. typhimurium* (8) and *S. anatum* (3) by. These two *Salmonella* ssp. were determined in the RapID™ ONE test as two different digit numbers: 4320000 probably indicates *S. typhimurium* and 6120010 indicates *S. anatum*.

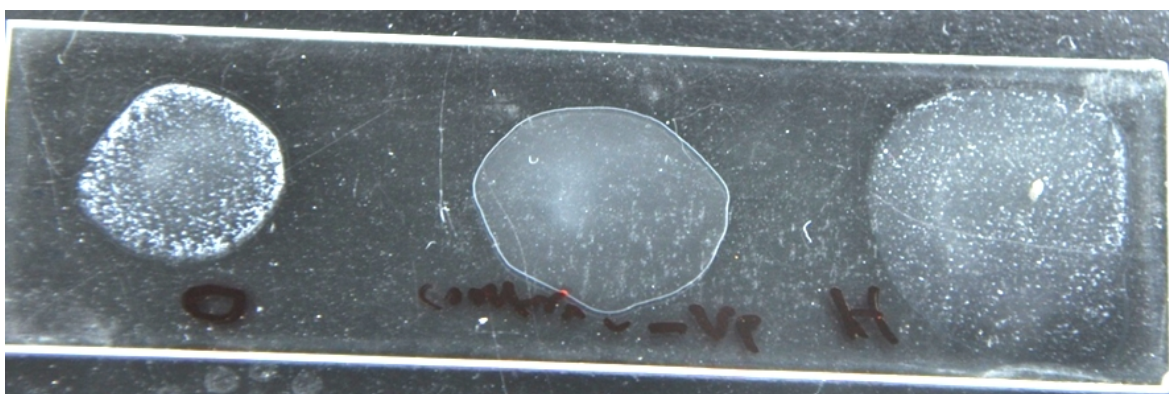


Figure 4.3. Agglutination tests for *Salmonella* with polyvalent O and H antisera

4.5. Antibiotic Susceptibility of *Salmonella*

Antibiotic resistance of *S. typhimurium* and *S. anatum* was tested for susceptibility with 13 kinds of antibiotics by the disk diffusion method in MH agar. Table 4.4 shows the result of antibiotic sensitivity of *Salmonella* ssp. Both *Salmonella* ssp. were sensitive to CT, C, CIP and TE. *S. anatum* also showed sensitivity to K and TMP.

Table 4.4. Antibiotic sensitivity of *Salmonella* ssp.

	Antibiotic	Abbreviation	Resistance	
			<i>S. typhimurium</i>	<i>S. anatum</i>
1	Amoxicillin/Clavulanic	AM	R	R
2	Carbenicillin	CT	S	S
3	Chloramphenicol	C	S	S
4	Ciprofloxacin	CIP	S	S
5	Doxycycline	DOC	I	I
6	Enrofloxacin	ENR	R	R
7	Erythromycin	E	I	I
8	Gentamicin	GN	R	R
9	Kanamycin	K	R	S
10	Nalidixic acid	NA	R	R
11	Nitrofurantoin	F	R	R
12	Tetracycline	TE	S	S
13	Trimethoprim	TMP	R	S

S, Sensitive; I, intermediate; R, Resistant

4.6. Seasonal Variation in *Salmonella* Prevalence

There was significant seasonal variation in *Salmonella* isolates. High prevalence was recorded in the summer (13.3%), and low in the winter (4%) (Table 4.3).

Table 4.5. Seasonal variation in *salmonella* prevalence

	No. of samples	No. of isolate	% of isolate
Summer	75	10	13.3
Winter	25	1	4

5. DISCUSSION

While it is widely acknowledged that *Salmonella* is the major cause of food borne illness in poultry and its products, epidemiological data are needed to inform public health authorities about the nature and size of the problem and to monitor trends over time. Raw poultry products are contaminated with harmful, pathogenic and spoilage bacteria by infected stocks, cross contamination, improper handling and storage or improper cooking of poultry, which can lead to human foodborne illness and loss of product shelf life (Myint, 2004). Food safety regulations on meat and poultry are important issues and there should be a global plan to address hazards by perfection the food safety at the animal production and intermediate stages before the slaughter plant, food safety during transportation, storage and retail sale, and to educate consumers to follow safe food handling practices such as proper storage, preparation, and cooking of meat and poultry products.

This is the first study on the isolation and antimicrobial resistance of *Salmonella* in fresh and frozen chicken carcasses sold in Duhok and Arbil, Northern Iraq. It is found an overall *Salmonella* prevalence of 11%. This rate is close to those reported by Al-Abidy and Hadad (2007) in Mosul, Iraq (9.72%), Ozbey and Ertas (2006) in Turkey (12%), Dahal (2007) in Bhutan (13%) and Maharjan *et al.* (2006) in Nepal (14.5%). However, the low prevalence of *Salmonella* in chicken carcasses was found compared to many other countries such as South Africa 19.2% (Van Nierop *et al.*, 2005), Bangladesh 26.6% (Akond *et al.*, 2012), Spain 35% (Dominguez *et al.*, 2002), Vietnam 53% (Van *et al.*, 2007). The difference in the finding may be due to the fact that the actual prevalence of *Salmonella* is low or the processing industry decontaminate the final products or the producers use antimicrobials in the production chain or may be due to the isolation technique and number of samples examined.

Caution should be taken when making direct comparison between prevalence estimates of the pathogenic bacteria because of the differences in methods, samples number, frequency and time of sampling, transport and storage of samples, and season (Nouichi and Hamdi, 2009).

Among serotypes observed in this study, *S. typhimurium* (8) was the most common followed by *S. anatum* (3).

Approaches to prevent and control salmonellosis in the food animal industry by various means such as improved biosecurity, vaccination, use of competitive exclusion products, and the introduction of novel immuno-potentiators with limited success has necessitated the use of antimicrobial chemotherapy in the treatment and control of Salmonellosis (Zhao *et al.*, 2007). The use of antimicrobials in food animals has resulted in the development of antimicrobial resistance (White *et al.*, 2001), through mutation and acquisition of resistance encoding genes (Fluit, 2005). The situation in Iraq may be exaggerated by easy accessibility of antimicrobials at a cheaper price and their extensive use in poultry production. Another major setback might be the quality and potency of locally produced antimicrobial drugs. Thus there is widespread availability and uncontrolled use of antibiotics.

The result of antimicrobial susceptibility in this study showed that *S. typhimurium* (8 isolates) was sensitive to CT, C, CIP and TE, and *S. anatum* (3 isolates) to CT, C, CIP, TE, K and TMP. This could be due to wide and varied use of antibiotic as treatment and feed additive in poultry. Excessive and inappropriate use of antibiotic in the rearing of farm animals of food chain represents a major factor in the emergence, persistence and spread of multi drugs resistant *Salmonella* (Cruchaga, *et al.*, 2001). These finding were concurrent to the observation in Iraq of Hadad and Jemel (1990), AL-Aboudi *et al.*, (1993), Al-Abidy and Hadad (2007) and Abdulrahman (2010) and in other countries of Duffy *et al.*, (1999), Dahal (2007), Smith *et al.* (2009), and Kaushik *et al.* (2014). All studies showed that *Salmonella* strains isolated from food and meat product of animal origin were multi drugs resistant.

There was a major variation in seasonal prevalence of *Salmonella* in summer and early winter months. A total of 75 samples were analyzed during the summer and 25 samples during the winter. High prevalence where was recorded in summer (13.3%), and low in winter (4%). The rate of contamination probably increase in high temperatures; the traditional slaughtering , transportation and poor storage also play an important role in the prevalence of *Salmonella* during the summers in the Northern Iraq. The consignment is transferred and further transported in conventional cars. The temperatures rise as high as 38°C during summer in northren Iraq. This might be due to the fact that prevalence of *Salmonella* is higher during hot and dry seasons. These finding were similar to those reported in Nepal by (Maharjan *et al.*, 2006), They found that the prevalence of

Salmonella was found the highest during the months of April and May, in a study in Bhutan, The isolation of *Salmonella* during winter and late spring was significantly different ($p<0.001$) (Dahal, 2007).

6. CONCLUSION

The present study was designed to isolate and confirm *Salmonella* in chicken meats collected in the local markets and butcheries in Duhok and Arbil, Northern Iraq. In this respect, one-hundred fresh (50) and frozen (50) chicken meats were analyzed by the cultural, biochemical and serological methods. The suspected cultures were later subjected to the antibiotic resistance test using 13 known antibiotics.

The cultural method for isolation of *Salmonella* including pre-enrichment on buffered peptone water, enrichment in RV broth and selective enrichment in XLD and ABC agars indicated that 11 agar plates were probably suspected with *Salmonella*. The biochemical confirmation tests performed on the KI agar, SC agar and urease broth confirmed that 8 of 11 suspected culture contained *Salmonella*. The RapID™ ONE test was further used for the confirmation. The RapID™ ONE test result showed that all 11 suspected cultures probably contaminated with *Salmonella*. Two different digit numbers (4320000 and 6120010) obtained from the RapID™ ONE computer program indicated that the suspected cultures contained two different *Salmonella* spp.

In order to confirm *Salmonella*, the suspected cultures were subjected to the serological tests. Using somatic (O) and flagellar (H) antisera, it was observed agglutination of the suspected cultures with polyvalent O and H antisera, indicating the suspected cultures contained *Salmonella*. The cultures were serotyped by the Central Health Laboratory, and confirmed that 8 of them were contaminated with *S. typhimurium*, and 3 of them with *S. anatum*.

The tests performed in the study showed that the prevalence of *Salmonella* is higher in Arbil than in Duhok; *S. typhimurium* is dominant compared to *S. anatum* in the meat samples and both show multi-drug resistance. The fresh meat samples had higher prevalence of salmonella spp. than the frozen meat samples. High prevalence where was recorded in summer and low in winter.

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